5. LM, SEM AND TEM INVESTIGATIONS ON PARTIALLY DEGRADED BOTRYOCOCCUS BRAUNII KÜTZ. COLONIES FROM HUNGARIAN UPPER TERTIARY OIL SHALE I.

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Abstract

During our newest experimental investigations on the *Botryococcus braunii* colonies isolated from Upper Pannonian oil shale from Pula nine kinds of experiments were carried out. For the partial degradation of the colonies 2-aminoethanol, $KMnO_4$, and merkaptoethanol were used. The results obtained by the different methods were compared. Particular attention was paid for the different organization levels of the biopolymer units of the wall of the colonies. The problems of the different methods are discussed.

Key words: Alginite, partial degradation, LM, SEM, TEM.

Introduction

The hydrocarbon producing colonies of the *Botryococcus* KÜTZING genus are very interesting and peculiar among the *Algae*. This was the reason that the taxonomic position was not always unanimous. According to FRITSCH (1935) and KISS (1939) this genus belongs to *Xanthophyceae, Heterochloridales*. WILLE (in ENGLER and PRANTL 1910) classed into the *Chlorophyta (Chlorococcales - Protococcales) Botryococcaceae*, PANKOW (1976) into the *Chlorococcales, Dictyosphaeraceae*. There are further comprehensive papers in this respect, e.g.: BATTEN and GRENFELL (1996). The most important papers concerning the basic morphology of these peculiar colonies are as follows: BLACKBURN and TEMPERLEY (1936), FRÉMY and DANGEARD (1938), CHADEFAUD and EMBERGER (1960), COMBAZ (in DURAND, 1980), LARGEAU, DERENNE, CLAIRAY et al. (1990), GLIKSON, LINDSAY and SAXBY (1989), VÉR (1994) and BATTEN and GRENFELL (1996). The most important morphological characteristic features may be summarized as follows: 1. The colonies are enveloped in mucilage (=Schleimmasse = mucilagineuse = enveloppe gelatineuse hyaline = hydrocarbon matrix). FRÉMY and DANGEARD (1938) established polymorphism at the *Botryococcus* colonies both living and fossil. E. NAGY (1969) emphasized also the problem of polymorphism what may be in consequence of the ontogenetical stages, ecologic factors, such as temperature, light intensity, water composition, pH, etc. 2. The cellular components of the colonies are pedunculus and cupula (cup). Two cupulae form one polipier following CHADEFAUD and EMBERGER (1960). 3. Developmental stages of *Botryococcus braunii*, both recent and fossil, were

published by GUY-OHLSON (1992). Autospores on the sides of the cups of young colonies were published by ERLSTRÖM, GUY-OHLSON and SIVHED (1994) from Jurassic-Cretaceous layers, using the SEM method. The organelles of the protoplasm were summarized by CHADEFAUD and EMBERGER (1960), and COMBAZ (1980). The LM wall structure based on TEMPERLEY (BLACKBURN and TEMPERLEY, 1936) and LARGEAU et al. (1990) was summarized by BATTEN and GRENFELL (1996) as follows: The cellulose wall is closed by the cell cap. The cellulose wall is surrounded by three cups, the outest is the first cup, the third cup is in contact with the cellulose wall. The first taxonomical problems of the oil bearing alga were summarized by NARAYANA RAO and MISRA (1949): "yellow bodies" BERTRAND and RENAULT (1892), after *Reinschia australis*, and *Pila*. Later BLACKBURN and TEMPERLEY (1936), HARRIS (1938), TRAVERSE (1955) and GRAY (1960) reviewed the morphology, the geologic and the geographic distribution of *Botryococcus*. The taxonomic problems of the genus *Botryococcus* and *Gloeocapsomorpha* were discussed by KOSANKE and MYERS (1986), COLLINSON et al. (1994), BATTEN and GRENFELL (1996) and WICANDER, FOSTER and REED (1996). They pointed out that the *Gloeocapsomorpha* may not be cogeneric with *Botryococcus*. The ecology of *Botryococcus braunii* KÜTZ. was summarized by VÉR (1994) as follows; p. 12:

"i. Occurrence in eutrophic fresh water or in humid soil (WILLE, in ENGLER and PRANTL, 1910) in Europe, North America and Africa. These colonies can also be present in salt lakes or in the water of marine lagoons.

ii. Stenotherm species after the paper of KISS (1939). The geological distribution of this kind of algue is extremely large. JARZEN (1978) writes the following, p. 32: '*Botryococcus* KÜTZING (Pl. 1, fig. 3) is a colonial green algae, whose colonies form irregular globose masses encasted in a heavy, often dark, cohered mucilage. TRAVERSE (1955) has reviewed the fossil occurrences of the genus and notes that the fossil record probably extends back at least to the Ordovician.' (Cf. NARAYANA RAO and MISRA, 1949).

'The hydrocarbon secreting alga *Botryococcus* has been identified in organic remains of sediments ranging from Precambrian to Recent,' (GLIKSON, LINDSAY and SAXBY, 1989, p. 595)."

The colonies which may form the oil shale (Alginite) layers and its importance in the kerogen was recognized very early. In this way the industrial importance of this kind of algal colonies intensive and exhaustive and multidisciplinary researches were carried out. Regarding the chemical composition the three cups are the most important. Previously the sporopollenin content was established. Later based on the new results several alterations were pointed out, such as the extreme chemical variability of the colony matrix by BRENNER (1998), and the terms PRB ("dominated by linear fatty monocarboxylic acids but also comprise a linear dicarboxylic acid and a "pseudo" isoprenoid acid and exhibit a substantial contribution of isoprenoid acids." By DERENNE, LARGEAU and CASADEVALL (1991, p. 597), botryococcene (isoprenoid hydrocarbons) respectively botryococcane were introduced. To this we cite the paper of BERKALOFF, CASADEVALL, LARGEAU, et al. (1983), LARGEAU, CASADEVALL, KADOURI and METZGER (1984), DERENNE, LARGEAU, CASADEVALL and BERKALOFF, (1989), DERENNE, LARGEAU, CASADEVALL and CONNAN (1988a,b) and TEMPLIER, DIESENDORF, LARGEAU and CASADEVALL (1992).

TEM pictures were published from untreated recent colonies of *Botryococcus braunii* by WOLF and COX (1981), BURNS (1982), KADOURI, DERENNE, LARGEAU, et al. (1988), DERENNE, LARGEAU, CASADEVALL and BERKALOFF (1989), TEMPLIER, LARGEAU, CASADEVALL and BERKALOFF (1992).

SEM data from untreated colonies by TEMPLIER, LARGEAU, CASADEVALL and BERKALOFF (1992), ERLSTRÖM and GUY-OHLSON (1994) and GUY-OHLSON and LINDSTRÖM (1994).

TEM results of fossil colonies without experiment were published by KEDVES (1983) and DERENNE, LARGEAU, HETÉNYI, et al. (1997). SEM data from Hungarian *Botryococcus* colonies were published by E. NAGY (1976), GUY-OHLSON and LINDQVIST (1990) from Cambrian-Ordovician age from Sweden, DERENNE, LARGEAU, HETÉNYI, et al. (1997) from the locality of Pula (Hungary). From Early Permian untreated *Botryococcus* colonies GUY-OHLSON (1992), and GUY-OHLSON and LINDSTRÖM (1994) published new data. Single, compound colonies and autospores were illustrated. The different stages of preservation were also discussed. Jurassic data from GUY-OHLSON (1996).

The presence of the *Botryococcus* colonies in the Hungarian sediments was first published by E. KRIVÁN-HUTTER (1963). Later several LM data were published from different localities and ages. After the discovery of a great amount of Alginite in Transdanubia in Hungary multidisciplinary researches started on this sediment. The most important results are as follows: The oil shale (Alginite) from Hungary was first discussed by JÁMBOR and SOLTI (1975). Geology: JÁMBOR (1980), HETÉNYI (1985), JÁMBOR and SOLTI (1975, 1976), SOLTI (1981). Geology and Mineralogy: MEZŐSI (1976), Petrography: RAVASZ (1976). Ecology: E. NAGY (1976), HAJÓS (1976), JÁMBOR (1980). Geochemistry: ARATÓ and BELLA (1976), HETÉNYI and VARSÁNYI (1976), GRASSELLY, BERTALAN and SAJGÓ (1977), HETÉNYI, MAITZ and TÓTH (1977), HETÉNYI (1979, 1980, 1985, 1987-1988), HETÉNYI and PÁPAY (1986), HETÉNYI and SIROKMÁN (1978), plant macrofossils: KVAČEK, HABLY and SZAKMÁNY (1994), palynology by E. NAGY (1965, 1975a,b, 1992, 1993, 1997).

The aim of this paper is to compare the results of the different methods, and to establish, whether the resolution of the used SEM instrument and the method is suitable to demonstrate the larger biopolymer units, which may be modelled by the fullerenes. We must emphasize the important methodical differences between the TEM and SEM instruments used. It may be presumed that the data of the SEM instruments of a resolution power more or less identical with the TEM instrument or at least below 10 Å, and without metal covering will bring more exact data in this respect, but we hope that our data obtained with the instruments of our present day opportunities will be useful for the further investigations on this really interesting and important subject.

Previous experimental investigations

KEDVES (1986a) published the first TEM results of the partially degraded colonies of *Botryococcus braunii* KÜTZ. isolated from Hungarian oil shale. Globular macromolecular units were observed, which may be arranged into filaments or are in irregular position. Results of combined investigations (LM, TEM and thin layer chromatography) were published by KEDVES (1986b). By the way of comparative thin layer chromatography molecular remnants of chlorophyllids, chlorophylls, carotenoids, ?lutein and two unidentifiable components between chlorophyllids and violaxanthin were established.

KEDVES (1987) concerning the studies of the degradation of the sporoderm under natural and in vitro conditions pointed out, that there are similarities between fossil angiosperm exines and the walls of fossil algae, e.g. *Botryococcus* and *Pleurozonaria*. KADOURI, DERENNE, LARGEAU, et al. (1988), DERENNE, LARGEAU, CASADEVALL and BERKALOFF (1989) published TEM picture of treated recent colonies for isolating PRB.

DUBREUIL, DERENNE, LARGEAU, BERKALOFF and ROUSSEAU (1989) published TEM data from the Darwin Coorongite and saponified *Botryococcus braunii* B race colonies. Concentric lamellae were described. DERENNE, METZGER, LARGEAU, et al. (1991) established similar morphological variations on *Gloeocapsomorpha prisca* in Ordovician sediments and cultured *Botryococcus braunii* in consequence of the changes in salinity.

TEMPLIER, LARGEAU, CASADEWALL and BERKALOFF (1992) published SEM and TEM pictures from untreated, after lipid extraction, after basic hydrolysis from the A and B races of *Botryococcus* colonies (recent). Characteristic lamellar structures of the wall are illustrated in picture Bi, Fig. 5., after lipid extraction. KEDVES, ROJIK and VÉR (1992) concerning the biopolymer organization of the *Botryococcus* colonies from Hungarian Alginite pointed out the following; p. 21: "The experiments were made in the following ways: 1. Partial dissolution and degradation of the colonies with NaOH, 2-aminoethanol, $KMnO_4$ aq. dil. and with combination of the two latter mentioned chemicals. 2. Protoplast method (HELIX enzymatic destruction)".

KEDVES, TÓTH and FARKAS (1993) emphasized that the large globular units described first by KEDVES, ROJIK and VÉR (1991) can be equated with the recently discovered fullerenes.

KEDVES, TÓTH and VÉR (1993) presented first the radial fivefold rotation, and the term of the rotation areas was introduced, and alterations and secularities of the rotation areas, and the importance of the extra-areal secondary points of symmetries were published as a preliminary report.

VÉR (1994) used the LM method to investigate the acceptance of different stains of the *Botryococcus* colonies from Pula, and she emphasized, that this method is suitable to establish the degrees of maturity of the organic components of the colonies.

KEDVES, TÓTH and VÉR (1993, 1995) published in detail the radial fivefold rotation method on the partially degraded quasi-periodic biopolymer structures of the wall of *Botryococcus* colonies isolated from the Alginite of Pula. For the first time two kinds of radial rotations were elaborated. In this paper the rotation area was also published secular alterations in the size and the shape of the rotation areas were established.

KEDVES, TRIPATHI, VÉR, PÁRDUTZ and ROJIK (1998) carried out new symmetry operations on a partially degraded and fragmented colony of *Botryococcus braunii* KÜTZ. On this fragment the quasi-periodic and quasi-equivalent biopolymer structures were established. Our pentagon biopolymer units were chosen from the bordering zone of these two kinds of symmetries. Different relationships were established between the quasi-crystalloid and quasi-equivalent biopolymer system. After partial degradation with 2-aminoethanol and merkaptoethanol for 30, 60, 90 and 150 days the molecular system sensu strictu was dissolved.

Materials and Methods

The investigation material came from Pula from the collection of Prof. Dr. M. HETÉNYI.

The partial dissolution and degradation experiments were made in the Cell Biological and Evolutionary Micropaleontological Laboratory as follows:

3 mg dry *Botryococcus* colonies were used for each experiment, at 30 °C temperature.

AKP-99-1. - 3 mg *Botryococcus* colony + 1 ml 2-aminoethanol, length of time 24 h.

AKP-99-2. - 3 mg *Botryococcus* colony + 1 ml 2-aminoethanol, length of time 48 h.

AKP-99-3. - 3 mg *Botryococcus* colony + 1 ml 2-aminoethanol, length of time 72 h.

AKP-99-4. - 3 mg *Botryococcus* colony + 1 ml 2-aminoethanol, length of time 24 h, after washing + 10 ml $KMnO_4$ 1%, length of time 24 h.

AKP-99-5. - 3 mg *Botryococcus* colony + 1 ml 2-aminoethanol, length of time 48 h, after washing + 10 ml $KMnO_4$ 1%, length of time 24 h.

AKP-99-6. - 3 mg *Botryococcus* colony + 1 ml 2-aminoethanol, length of time 72 h, after washing + 10 ml $KMnO_4$ 1%, length of time 24 h.

AKP-99-7. - 3 mg *Botryococcus* colony + 1 ml 2-aminoethanol, length of time 24 h, after washing + 1 ml merkaptoethanol, length of time 24 h.

AKP-99-8. - 3 mg *Botryococcus* colony + 1 ml 2-aminoethanol, length of time 48 h, after washing + 1 ml merkaptoethanol, length of time 24 h.

AKP-99-9. - 3 mg *Botryococcus* colony + 1 ml 2-aminoethanol, length of time 72 h, after washing + 1 ml merkaptoethanol, length of time 24 h.

For LM investigations the colonies were mounted in glycerine-jelly, hydrated at 39.6%. For SEM investigations the dry organic material was covered with gold-palladium, the pictures were taken in the SEM Laboratory of the Department of Botany of the University of Szeged on a Hitachi S-2400 instrument, resolution about 40 Å. For TEM investigations the partially dissolved and degraded colonies were embedded in Araldite without fixation with OsO_4 aq. dil. The TEM studies were made in the EM Laboratory of the Institute of Biophysics of the Biological Research Center of the Hungarian Academy of Sciences. For the ultrathin sections Porter Blum ultramicrotome with glass knives was used. The TEM photographs were taken on a Zeiss EM-902 (resolution 2-3 Å) and on a Tesla BS-540, resolution 6-7 Å.

Results

In Plate 5.1. non-experimental LM (fig. 1) and TEM (fig. 2) picture represents the basic morphology and ultrastructure of the *Botryococcus braunii* colonies isolated from the alginite of Pula. The light microscopical picture well illustrate the relatively long pedunculus and the cupules. The basic ultrastructure of the well preserved non-experimental wall is compact with tiny holes and sometimes differences in the electron affinity of the outer wall are illustrated (Plate 5.1., fig. 2). To the latter mentioned picture on the negative of a previously published (KEDVES, 1983) matter was used.

As a basic comprehensive material we publish again a TEM picture (Plate 5.2.) of the partially degraded and fragmented wall of the *Botryococcus* colonies, which was first published by KEDVES, ROJIK and VÉR (1991). Later it was pointed out that the large globular biopolymer units may be modelled with the fullerenes.

The diameter of the smaller globular units was measured recently, the results are as follows:

20	24	28	32	36	40	44	48	52	56	60	Å
21.4	20.0	12.9	11.4	7.0	8.6	3.6	5.7	2.9	3.6	2.9	%

The diameter of the large globular units is about 224 - 240 Å.

1. LM results

Experiments: AKP-99-1-3 (Plate 5.3., figs. 1-3)

After partial dissolution with 2-aminoethanol during 1-2-3 days no important alterations were established in the light microscopical morphology. The only thing is, that this method was not suitable to demonstrate the presence of the characteristic mucilage on the surface.

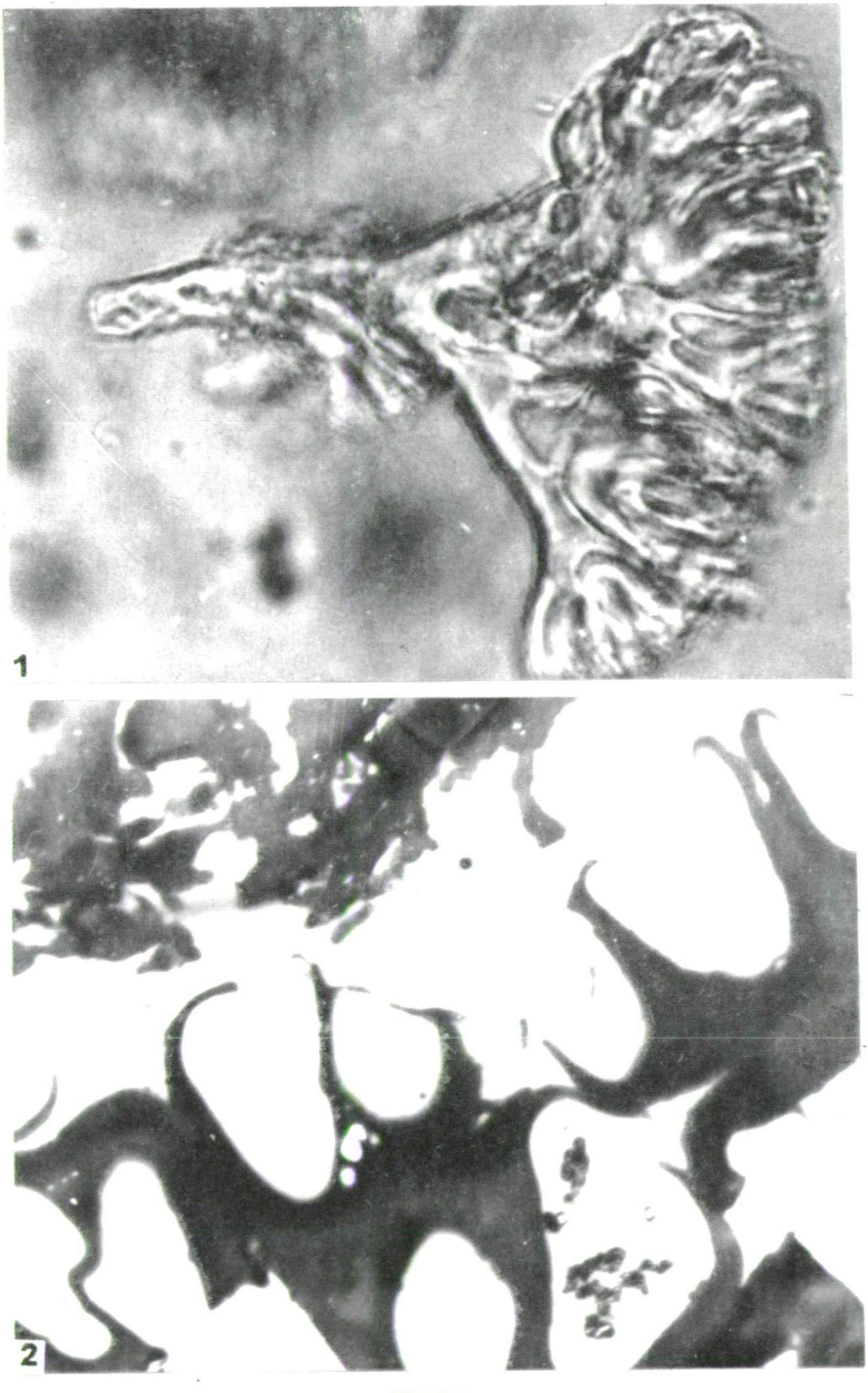


Plate 5.1.

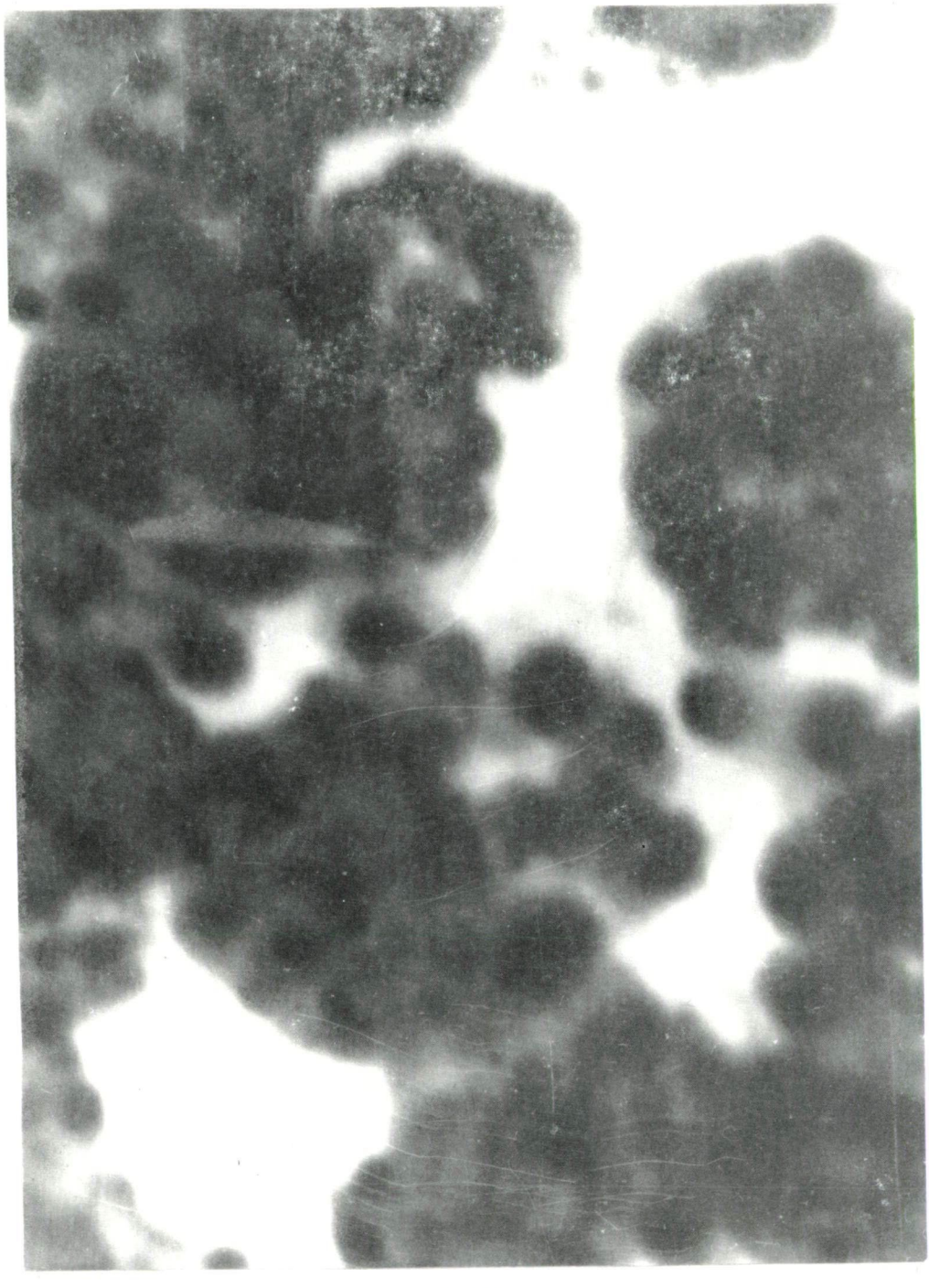


Plate 5.2.

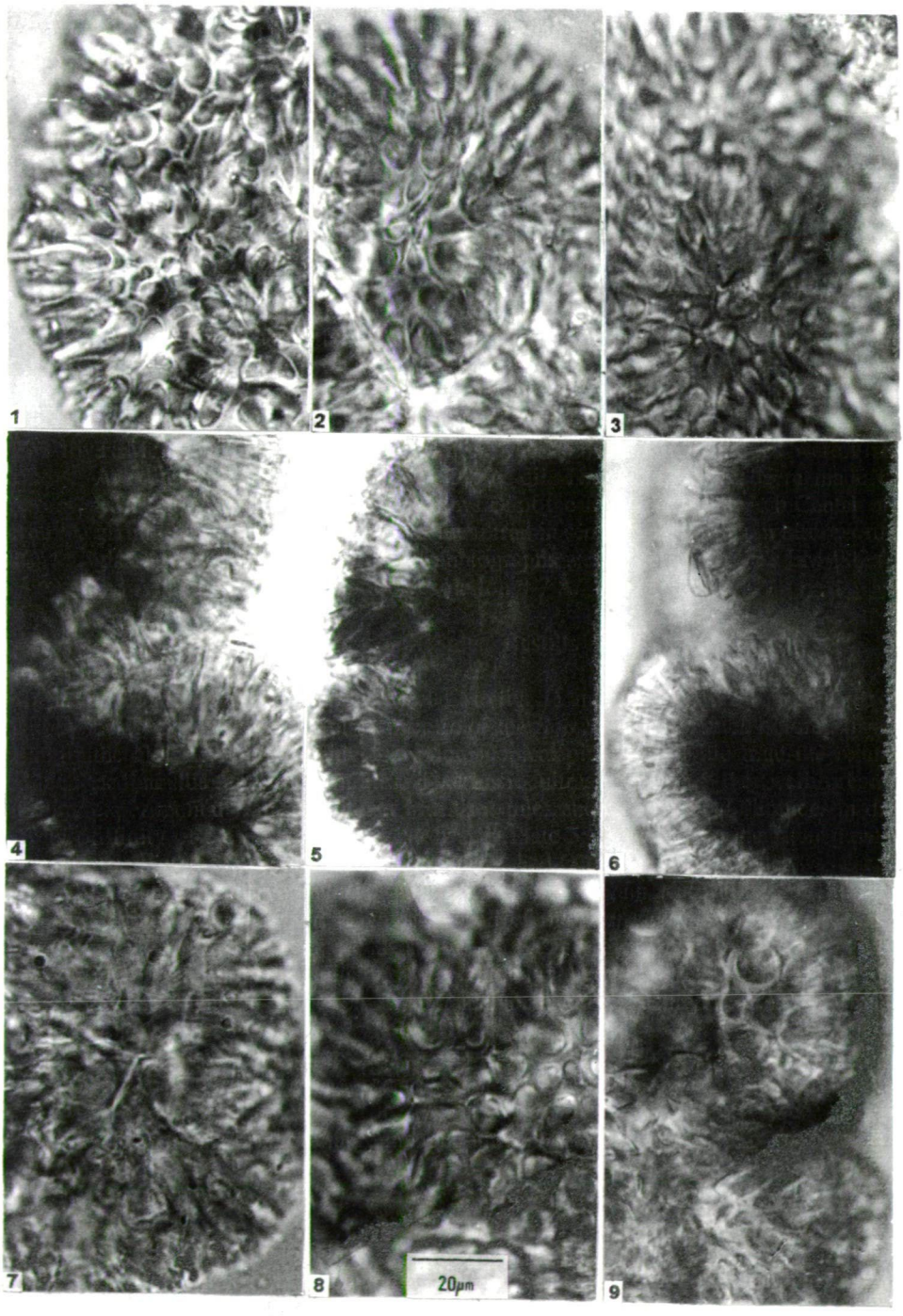


Plate 5.3.

Experiments: AKP-99-4-6 (Plate 5.3., figs. 4-6)

The oxidation after partial dissolution with 2-aminoethanol resulted in important alterations in the light microscopy of the colonies. The gradual degradation of the outer part of the colonies is well shown in pictures 4,5,6 in Plate 5.3. The inner part of the colonies is very dark, sometimes dark granules are present in the outside cupules of the colonies.

Experiments: AKP-99-7-9 (Plate 5.3., figs. 7-9)

The effect of the merkaptoethanol after 2-aminoethanol is well shown by the LM method too. A peculiar dissolution may be established, the walls of the cupules is not so characteristic in comparison with the experiment AKP-99-1 (Plate 5.3., fig. 1). Electron dense particles may be observed in the cupules in picture 7 of Plate 5.3.

2. EM results

Experiment: AKP-99-1 (Plate 5.4., figs. 1,2, plate 5.5., figs. 1,2)

SEM pictures (Plate 5.4., figs. 1,2) illustrate well the surface of the cupulae and the outer part of the wall. Remnants of the mucilage are well shown. Alterations in the ultrastructure of the wall were established with the transmission electron microscopical method (Plate 5.5., figs. 1,2). In the picture of low magnification (Plate 5.5., fig. 1) different kinds of electron dense particles were observed. These are granular, units separated or in linear or irregular arrangement (Plate 5.5., fig. 2). The general survey of this substance of the partially degraded wall is spongy.

Plate 5.1.

1,2. *Botryococcus braunii* KÜTZ. non experimental colonies.
1. LM picture of a colony. Illustrated are the relatively long pedunculus and the lateral view of the cupules. Slide: Pula-II-00-1, 1.200x.
2. TEM picture of the colony. Negative No: 3009, 2.500x.

Plate 5.2.

Biopolymer structure of the partially degraded and fragmented wall of *Botryococcus braunii* KÜTZ. Experiment No: 925, negative No: 0596, 250.000x.

Plate 5.3.

1-9. *Botryococcus braunii* KÜTZ. LM pictures of the partially degraded colonies.
1. Experiment No: AKP-99-1.
2. Experiment No: AKP-99-2.
3. Experiment No: AKP-99-3.
4. Experiment No: AKP-99-4.
5. Experiment No: AKP-99-5.
6. Experiment No: AKP-99-6.
7. Experiment No: AKP-99-7.
8. Experiment No: AKP-99-8.
9. Experiment No: AKP-99-9.

Experiment: AKP-99-2 (Plate 5.4., figs. 3-5, plate 5.5., figs. 3,4).

The magnified SEM pictures (Plate 5.4., figs. 4,5) illustrate globular units on the surface. The diameter of these units is as follows:

20	30	40	50	60	70	80	90	100	Å
28.6	22.1	7.8	14.3	7.8	6.5	9.0	2.6	1.3	%

It seems that based on the diameter the superficial globular units may be classed into two groups: 1. 20-30 Å, 2. 50-100 Å. This difference is well illustrated in picture 5, Plate 5.4. In the TEM pictures within the cupules electron dense particles were observed which may be remnants of the kerogens, e.g. fig. 3, in Plate 5.5. Not characteristic lamellar structure was also observed. On the highly magnified pictures (Plate 5.5., fig. 4) electron dense granular structures were also observed in the less electron dense substance.

Experiment: AKP-99-3 (Plate 5.4., figs. 6-8, plate 5.5., figs. 5,6)

In the highly magnified SEM pictures lamellar structures in the wall of the cupules (Plate 5.4., fig. 7) and destruction of the superficial layers were observed (Plate 5.4., fig. 8). Characteristic superficial globular units were not observed in contrast to the previous experiment. Based on the transmission electronmicroscopical results the degradation of the wall is advanced (Plate 5.5., figs. 5,6). There are globular electron dense particles in the wall of the cups (Plate 5.5., fig. 6).

Experiment: AKP-99-4 (Plate 5.6., figs. 1-3, plate 5.7., figs. 1,2)

The surface of the cups is more or less smooth, with remnants of the mucilage or other organic debris (Plate 5.6., fig. 3). Occasionally not so characteristic lamellar wall structure was observed (Plate 5.6., fig. 2). The lamellar structure of the wall is well illustrated on the TEM pictures (Plate 5.7., fig. 1). There are differences in the electron density of the lamellae. The different organization levels of the molecular structure are well shown in the highly magnified pictures (Plate 5.7., fig. 2). The per cents of the diameter of the globular units are as follows:

2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	Å
1.4	19.8	18.0	27.0	9.5	6.7	4.0	-	-	-	2.7	4.0	2.7	-	-	-	-	1.4	1.4	-	-	1.4	%

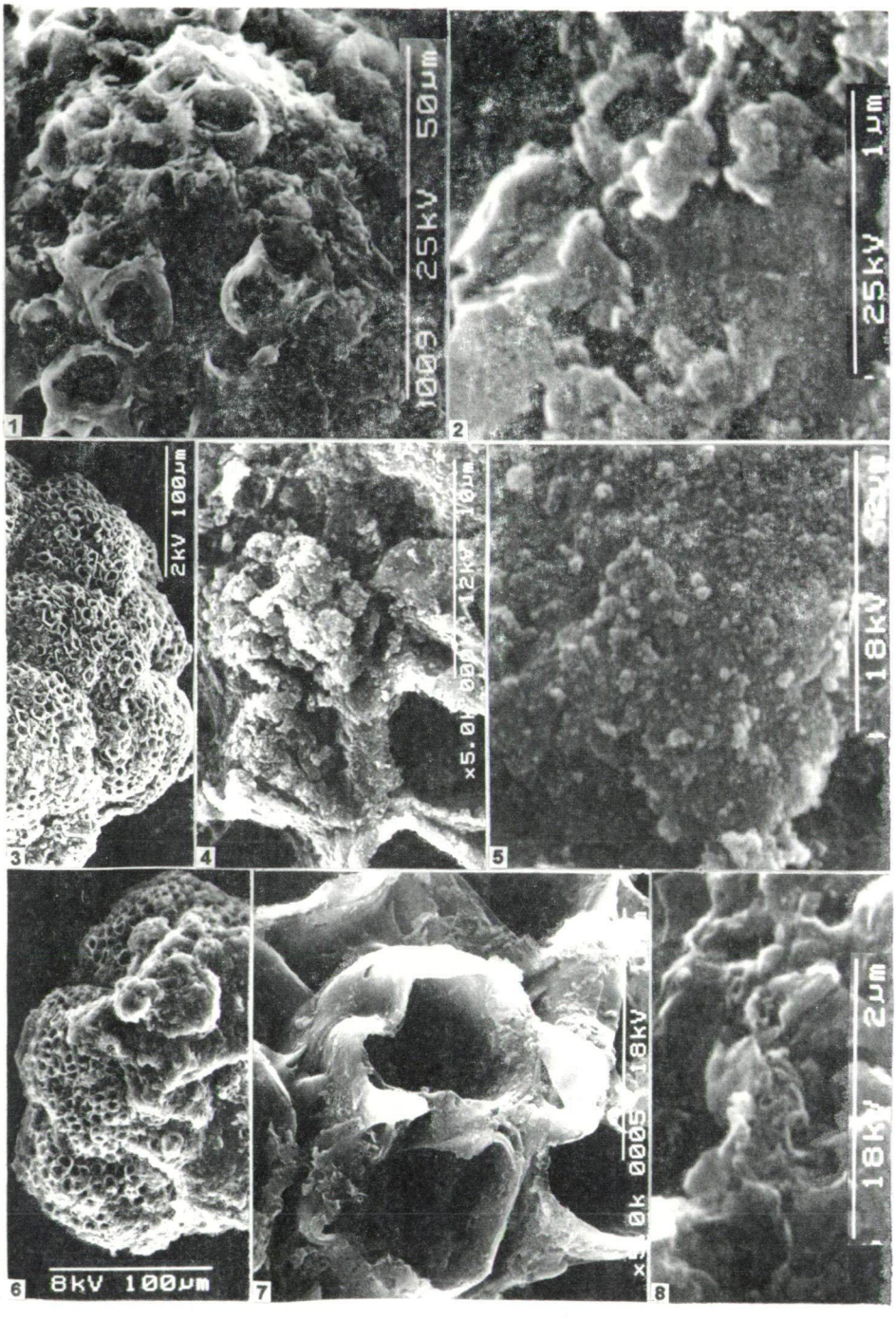
This experiments was the best for molecular symmetry operations, which will be made in the future.

Experiment: AKP-99-5 (Plate 5.6., figs. 4-6, plate 5.7., figs. 3,4)

Based on the SEM data the lamellar structure of the wall is not characteristic. (Plate 5.6., fig. 5). In the highly magnified SEM pictures the larger globular molecular units are well presented.

The percentages of the different diameter of the globular units are the following:

20	30	40	50	60	Å
16.5	42.2	29.3	11.2	0.8	%


1
1009 25kV 50µm
2
25kV 1µm
3
2kV 100µm
4
5
18kV
6
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7
8
18kV 2µm

Plate 5.4.

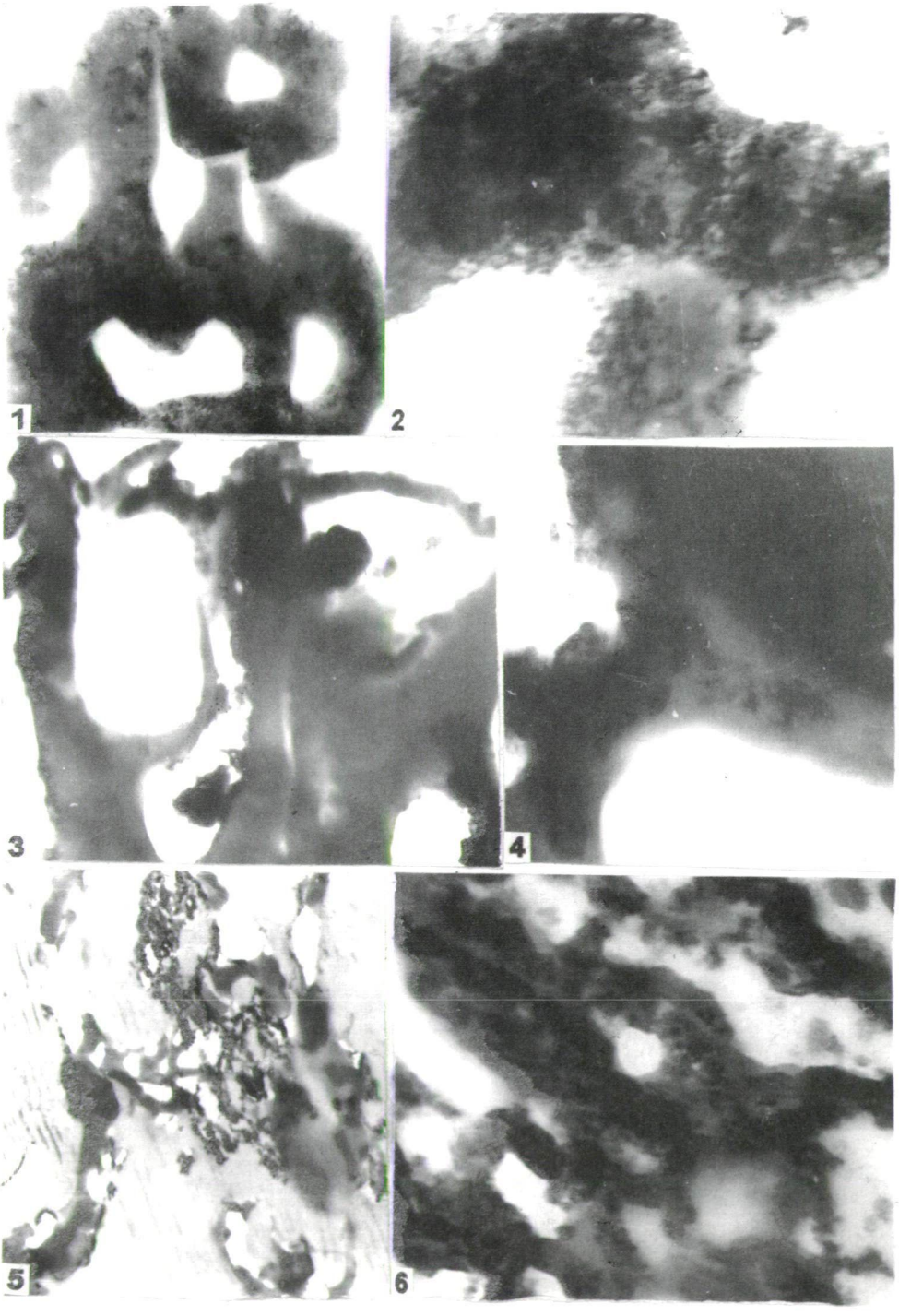

Plate 5.5.

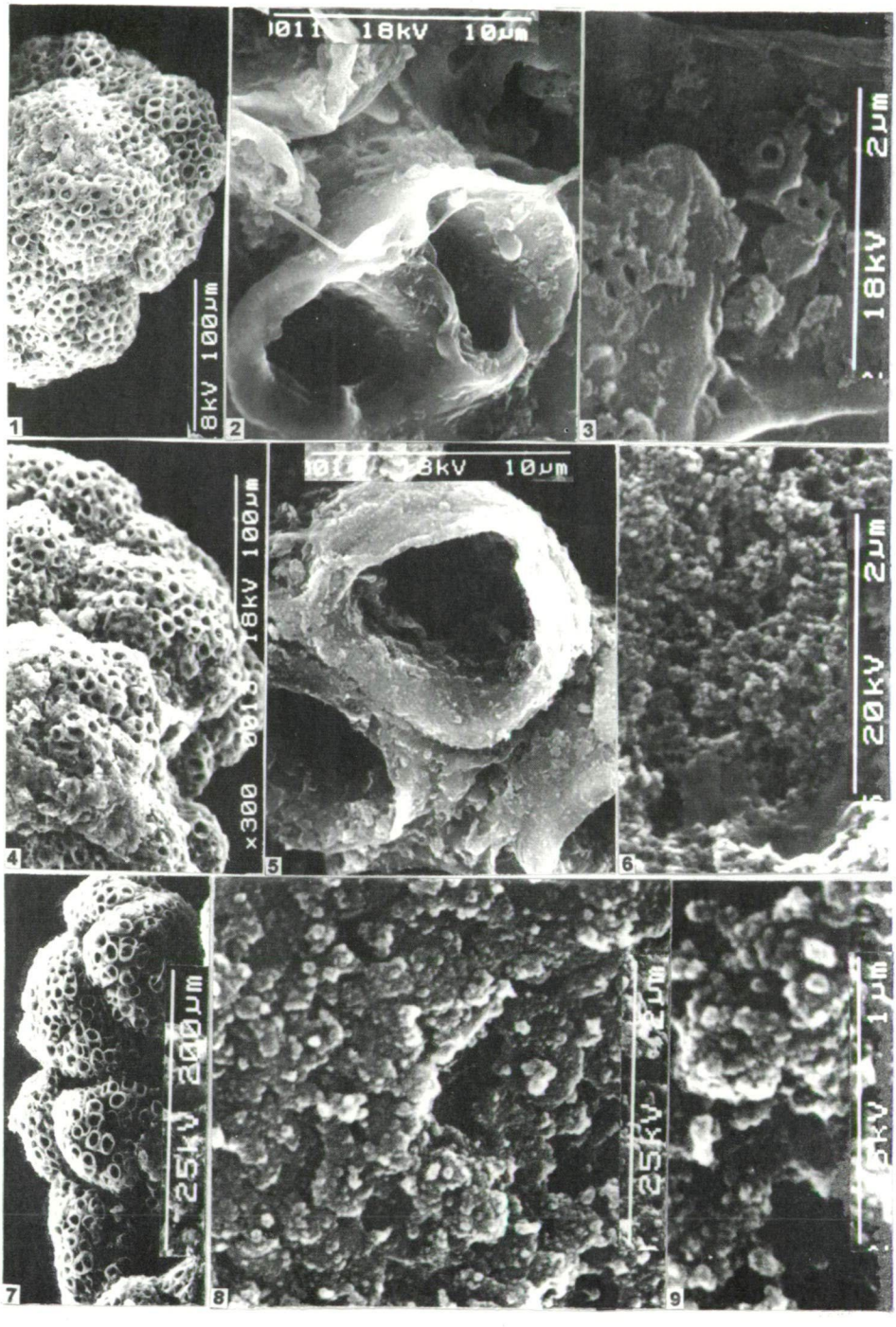
1
8kV 100µm
2
18kV 10µm
3
18kV 2µm
4
×300
18kV 100µm
5
10µm
6
20kV 2µm
7
25kV 200µm
8
25kV
9
1µm

Plate 5.6.

Plate 5.4.

1-8. *Botryococcus braunii* KÜTZ. SEM. pictures of the partially degraded colonies.
1,2. Experiment No: AKP-99-1.
3-5. Experiment No: AKP-99-2.
6-8. Experiment No: AKP-99-3.

Plate 5.5.

1-6. *Botryococcus braunii* KÜTZ. TEM pictures of the partially degraded colonies.
1,2. Experiment No: AKP-99-1. 1. Negative No: 7085, 15.000x, 2. Negative No: 7806, 50.000x.
3,4. Experiment No: AKP-99-2. 3. Negative No: 7773, 5.000x, 4. Negative No: 7774, 15.000x.
5,6. Experiment No: AKP-99-3. 5. Negative No: 7778, 5.000x, 6. Negative No: 7776, 50.000x.

Plate 5.6.

1-9. *Botryococcus braunii* KÜTZ. SEM pictures of the partially degraded colonies.
1-3. Experiment No: AKP-99-4.
4-6. Experiment No: AKP-99-5.
7-9. Experiment No: AKP-99-6.

The TEM pictures illustrate well the characteristic lamellar ultrastructure of the wall (Plate 5.7., fig. 3). The kerogen content of the cup was also observed. There are differences in the electron density in this organic content. Worth of mentioning is, the degradation of the molecular system (Plate, 5.7., fig. 4). The diameters of the more or less well preserved globular units are as follows:

2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	Å
11.5	16.3	21.2	24.0	2.9	4.8	5.8	3.8	3.8	1.0	-	-	-	1.0	1.9	-	1.0	1.0	%

Experiment: AKP-99-6 (Plate 5.6., figs. 7-9, plate 5.7., figs. 5,6)

Autospores are illustrated in the general survey SEM pictures (Plate 5.6., fig. 7). The globular biopolymer structures of the surface are well shown in the highly magnified SEM pictures. The percentages in the size are as follows:

20	30	40	50	60	70	80	90	100	110	120	Å
13.3	26.2	18.9	15.1	12.0	6.9	4.4	1.3	1.3	-	0.6	%

The general survey TEM picture illustrates well the lamellar system of the cups. The electron density of the different lamelles are characteristic. In some part of the ultrathin sections globular biopolymer units of different size were measured. The percentages of the diameter of the smaller globular units are as follows:

20	30	40	50	60	70	Å
0.5	14.1	36.7	35.0	9.2	4.5	%

Based on the measurement of few large globular units, the diameter is about 210-250 Å.

Experiment: AKP-99-7 (Plate 5.8., figs. 1-3, plate 5.9., figs. 1,2)

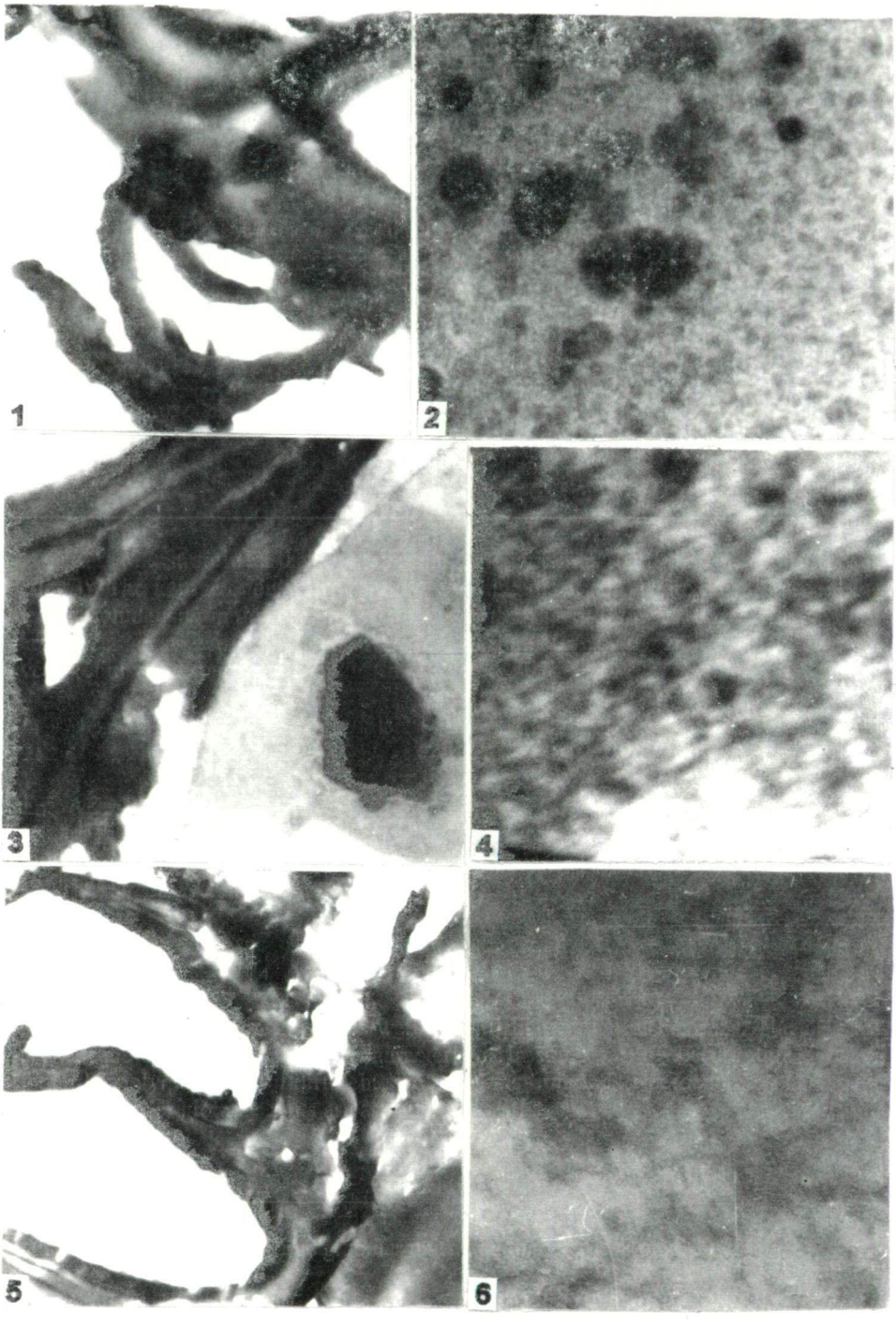

Plate 5.7.

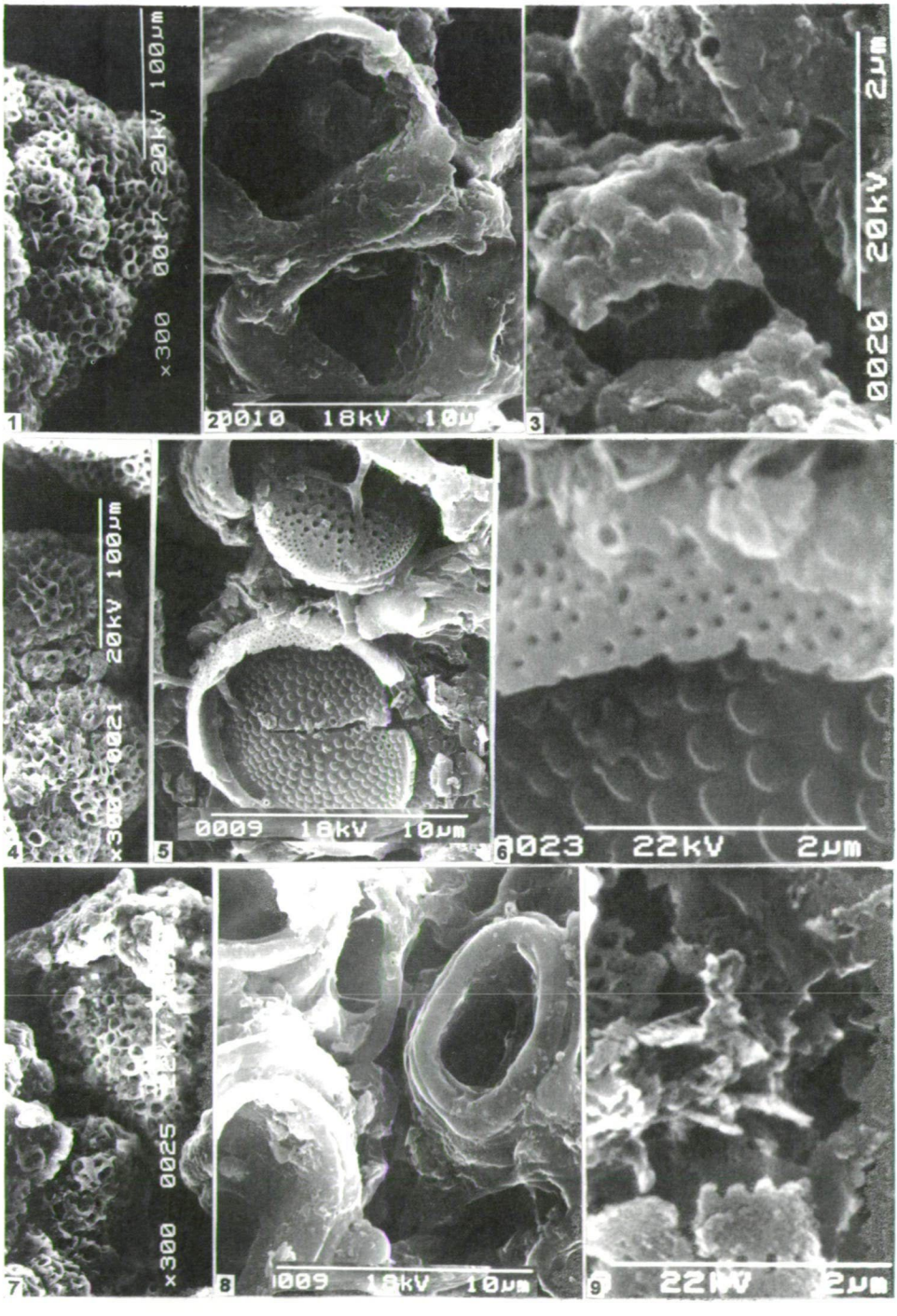
1
×300 0017 20kV 100µm
2
0010 18kV 10µm
3
0020 20kV 2µm
4
×300 0021 20kV 100µm
5
0009 18kV 10µm
6
0023 22kV 2µm
7
×300 0025
8
1009 18kV 10µm
9
22kV 2µm

Plate 5.8.

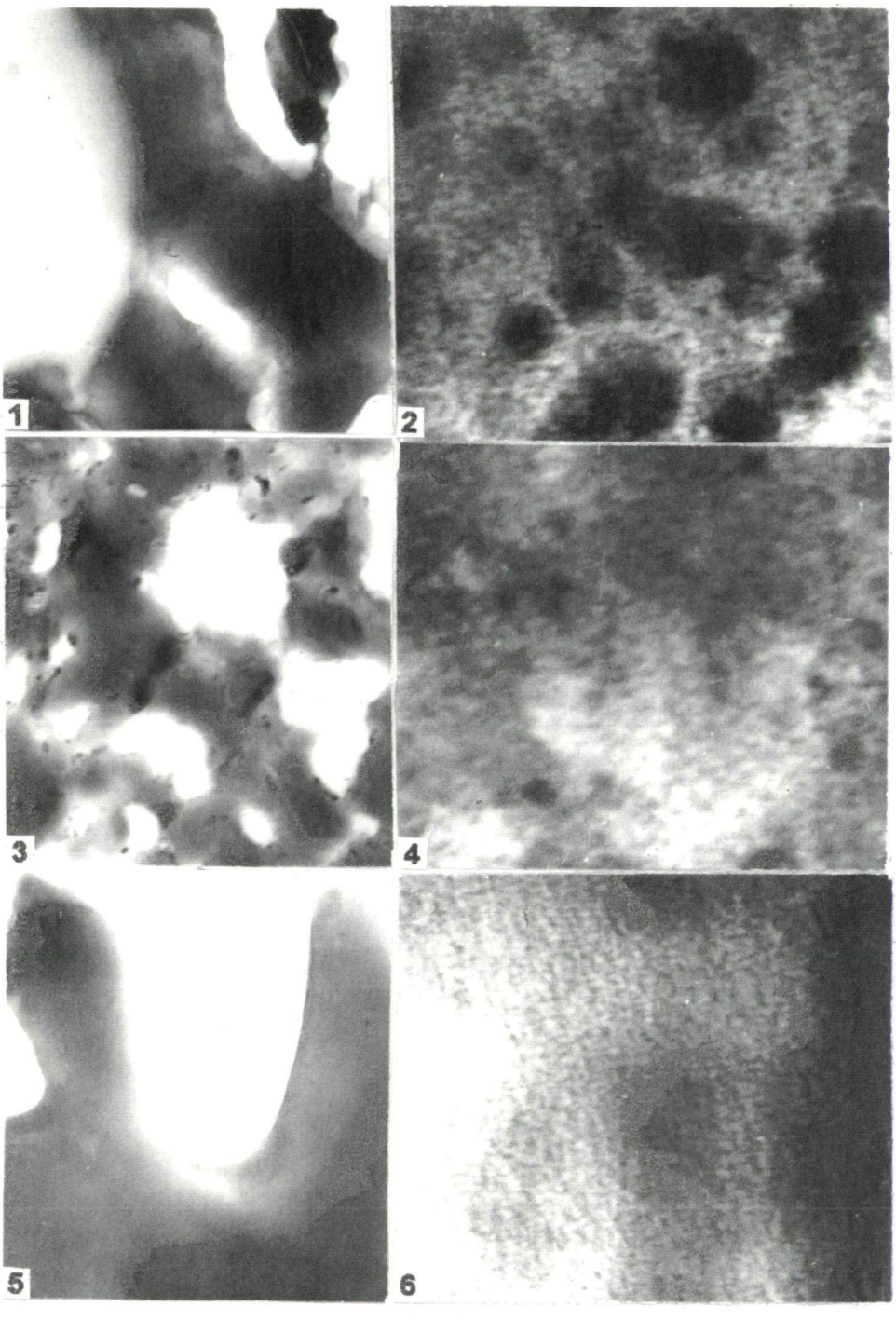

Plate 5.9.

Autospores are illustrated in the general survey SEM pictures (Plate 5.8., fig. 1). The highly magnified superficial pictures illustrate more or less smooth surface with some organic debris (Plate 5.8., figs. 2,3). In the low magnified TEM picture more or less homogeneous wall was observed (Plate, 5.9., fig. 1). Highly magnified TEM picture revealed the different kinds of molecular systems. The size of the globular units is as follows:

30	40	50	60	70	80	90	100	110	120	130	140	150	160	170	180	190	200	210	220	Å
10.0	6.0	12.0	10.0	2.0	10.0	10.0	4.0	12.0	4.0	12.0	-	2.0	-	-	-	-	4.0	-	2.0	%

Experiment: AKP-99-8 (Plate 5.8., figs. 4-6, plate 5.9., figs. 3,4)

Autospores are well seen in the general survey SEM picture (Plate 5,8., fig. 4). In the highly magnified pictures perforations in the inner, third cup were observed. The diameter of these perforations is as follows:

20	30	40	50	60	70	Å
9.7	20.2	41.1	21.0	6.4	1.6	%

The superficial ornamentation of the cell cup or the cup content are illustrated in picture 5 and 6, plate 5.8. The diameter of the ornamental elements is 150 - 290 Å, average 216 Å.

There are perforations on the ornamental elements of 10 - 20 Å in size.

The general survey TEM picture illustrates a more or less homogeneous wall with small electron dense particles. There are globular or elongated electron dense small particles (Plate 5.9., fig. 3). In the highly magnified picture globular biopolymer units are well presented, the degradation of the larger globular units is well shown (Plate 5.9., fig. 4).

20	30	40	50	60	70	80	90	100	110	120	130	140	150	160	170	180	190	200	210	220	Å
7	34	22	11	10	3	4	3	3	-	-	1	-	-	-	1	-	-	-	-	1	%

Plate 5.7.

1-6. *Botryococcus braunii* KÜTZ. TEM pictures of the partially degraded colonies.
1,2. Experiment No: AKP-99-4. 1. Negative No: 7769, 15.000x. 2. Negative No: 8623, 500.000x.
3,4. Experiment No: AKP-99-5. 3. Negative No: 7767, 15.000x. 4. Negative No: 8620, 1.000.000x.
5,6. Experiment No: AKP-99-6. 5. Negative No: 7784, 5,000x. 6. Negative No: 7751, 100.000x.

Plate 5.8.

1-9. *Botryococcus braunii* KÜTZ. SEM pictures of the partially degraded colonies.
1-3. Experiment No: AKP-99-7.
4-6. Experiment No: AKP-99-8.
7-9. Experiment No: AKP-99-9.

Plate 5.9.

1-6. *Botryococcus braunii* KÜTZ. TEM pictures of the partially degraded colonies.
1,2. Experiment No: AKP-99-7. 1. Negative No: 7763, 15.000x. 2. Negative No: 8616, 1,000.000x.
3,4. Experiment No: AKP-99-8. 3. Negative No: 7759, 15.000x. 4. Negative No: 8612, 1,000.000x.
5,6. Experiment No: AKP-99-9. 5. Negative No: 7754, 50.000x. 6. Negative No: 8608, 1,000.000x.

Experiment: AKP-99-9 (Plate 5.8., figs. 7-9, plate 5.9., figs. 5,6)

Autospores are illustrated in the general survey SEM picture (Plate 5.8., fig. 7). Not so characteristic lamellar structure of the cups and different kinds of organic remnants on the surface are illustrated in the highly magnified SEM pictures (Plate 5.8., figs. 8,9). The degradation of the biopolymer structure of the wall may be emphasized on the basis of the highly magnified TEM picture (Plate 5.9., fig. 6).

Discussion and Conclusions

1. In the first place it is necessary to emphasize the heterogeneous character of our experimental material. Differences are in the ontogenetic stages of the colonies, in this way the molecular system is different, consequently the alterations during the sedimentation processes are also different. Of course this has an effect on the results of the experiments. This problem was emphasized by GUY-OHLSON and LINDQVIST (1990), too, and illustrated with SEM pictures from *Botryococcus* colonies of different stages of development and state of preservation. The new results concerning the molecular system are also worth of mentioning at this question, namely the biopolymer of these *Algae* is named PRB (Polymère Resistant de *Botryococcus*) LARGEAU, CASADEVALL, KADOURI and METZGER (1984), DERENNE, LARGEAU and CASADEVALL (1991), algaenan; cf. TEGELAAR, DE LEEUW, DERENNE and LARGEAU (1989), LARGEAU, DERENNE, CASADEVALL et al. (1990), BRENNER, (1998), DE LEEUW, VAN BERGEN et al. (1991) botryococcene highly unsatured isoprenoid hydrocarbons (DUBREUIL, DERENNE, LARGEAU et al. (1989), respectively botryococcane (fossil biopolymer) to distinguish from the other sporopollenin type biopolymer systems (e.g.: DERENNE, LARGEAU, CASADEVALL and CONNAN (1988 a,b), DUBREUIL, DERENNE, LARGEAU et al. (1989), BRENNER (1998), etc.).

2. Our LM results on the non-coloured colonies may be summarized as follows: The 2-aminoethanol (experiments AKP-99-1-3) "cleaned" the surface of the cups. The oxidation with $KMnO_4$ (experiments AKP-99-4-6) revealed dark content within the cups. Finally the experiments AKP-99-7-9, by 2-aminoethanol and merkaptoethanol resulted in not so characteristic degradations on the cups.

3. As regards the EM results in the first place it is necessary to emphasize that there are very important methodical differences and problems. The TEM pictures were taken with instruments of 6-7 Å, respectively 2-3 Å, and the colonies were embedded without OsO_4 aq. dil. postfixation. The resolution of the used SEM instrument is about 40 Å, and the material of investigations was covered with gold-palladium. We need to point out these basic methodical problems, together with the heterogeneous character of our experimental material, however we try to start the evaluation of the data obtained by different methods. We know well that the present day conclusions will be modified or changed in the future based on the new SEM data of instrument with more much better resolution (below 10 Å) and without metal covering.

3.1. SEM results:

3.1.1. The degradation of the mucilage started with the dissolution with 2-aminoethanol during 24 h.

3.1.2. Superficial lamellae respectively and different cups respectively were observed at different kinds of experiments (Plate 5.4., fig. 7, plate 5.6., fig. 2, plate 5.8., fig. 8). It seems that in this case the maturity or the diagenesis of the colonies is an important factor in this result. DERENNE, LARGEAU, HETÉNYI et al. (1997) based on the SEM in-

vestigations of the untreated colonies of *Botryococcus braunii* from Pula distinguished thick multilayered, and thinner and less layered outer walls.

3.1.3. Larger biopolymer globular systems were observed in the first place after the combined degradation with 2-aminoethanol and $KMnO_4$ (Plate 5.6., figs. 6,8,9) but occasionally with 2-aminoethanol only during 48 hours (Plate 5.4., fig. 5). Perforations of the cup and ornamentation of the surface of the probably kerogen content of the cell cup was observed till this time at one experiment.

3.2. TEM results

Characteristic lamellar ultrastructure of different electron density was observed after experiments with 2-aminoethanol and $KMnO_4$ (Plate 5.7., figs. 1,3,5). The organization of the cups (first, second and third) may be well recognized (cf. BATTEN and GRENFELL, 1996). Different kinds of electron dense particles were observed within the wall of the cups in the first place at the experiments AKP-99-1-3, and 8.

4. Evaluation of the different organization levels of the globular biopolymer structures investigated by different methods.

4.1. The largest globular structures of diameter more than 130 Å.

4.1.1. TEM data of partially degraded and fragmented colonies (KEDVES, ROJIK and VÉR, 1991): 224-240 Å.

4.1.2. TEM data of the ultrathin sections of experiment AKP-99-6: 210-250 Å.

4.1.3. TEM data of the ultrathin sections of experiment AKP-99-7: 150-220 Å.

4.1.4. TEM data of the ultrathin sections of experiment AKP-99-8: 130-220 Å.

4.1.5. SEM data of experiment AKP-99-8: 150-290 Å.

4.1.6. For comparison we use the SEM data of the protectum of *Caesalpinia japonica* published by TAKAHASHI (1993, fig. 7, p. 196). The diameter of the globular units is as follows:

142.9	190.5	238.1	285.7	333.3	380.9	Å
14.1	38.8	24.8	14.9	4.1	3.3	%

These sporopollenin units are more or less in the category of our above discussed botryococcane biopolymer unit.

4.2. Globular structures of 20-130 Å diameter.

4.2.1. TEM data of the partially degraded and fragmented colonies (KEDVES, ROJIK and VÉR, 1991): 20-60 Å. These units are building elements of the above mentioned larger globular units.

4.2.2. TEM data of the ultrathin sections of experiment AKP-99-7: 30-130 Å.

4.2.3. SEM data of experiment AKP-99-2: 20-100 Å.

4.2.4. SEM data of experiment AKP-99-5: 20-60 Å.

4.2.5. SEM data of experiment AKP-99-6: 20-120 Å.

4.3. Globular structure of 2-20 Å.

4.3.1. TEM data of the ultrathin sections of experiment AKP-99-4: 2-23 Å.

4.3.2. TEM data of the ultrathin sections of experiment AKP-99-5: 2-19 Å.

The investigation of the further experiments is in progress. Trends in the alterations of the biopolymer structures may be established based on the following data. New symmetry operations are necessary to better understand this complicated biopolymer system.

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